Ciba Specialty Chemicals Corporation
North America
Performance Polymers

8EHQ-0997-13795 655

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August 27, 1997 TDW: 88970000045

CERTIFIED MAIL

Attn: Section 8(e) Coordinator
Document Processing Center (7407)
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
401 M Street, SW
Washington, D.C. 20460

8EHQ-96-13795

Re: TSCA Section 8(e) Notice: Supplemental Submission for 8EHQ-96-13795: Mechanistic Studies on Araldite® CY 179 and CY 184

Ciba Specialty Chemicals claims no information in this letter as Confidential Business Information.

In accordance with the EPA policy statement and reporting guide on TSCA Section 8(e), Ciba Specialty Chemicals is submitting the enclosed report on the investigation of the alkylation potency and epoxide hydrolysis of our products, Araldite CY 179, CY 184 and MY 790. This is a follow-up to our preliminary information submission on October 28, 1996, which was assigned the identification number of 8EHQ-96-13795 by the agency. The information and actions with the previous submission should be still applicable to this new submission.

Please contact me if you need further information.

Sincerely,

Jonas Weiss

Contains No CBI

Dr. Jonas Weiss
Director, Product Safety

Enclosure: (1)

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281 Fields Lane
Brewster, New York 10509

Tel. 914 785 3000

Value beyond chemistry

CY 179, CY 184 and MY 790

Investigation of Alkylation Potency and Epoxide Hydrolysis

FINAL REPORT
CB93/25-A

by
P. Sagelsdorff, B. Heuberger and G. Buser
Toxicology Services / Cell Biology
Novartis[1] Crop Protection Inc.
CH-4002 Basel
Switzerland

for
Ciba Speciality Chemicals
Performance Polymers Division
CH-4002 Basel
Switzerland

Date of Issue: January 31, 1997

This Report contains 27 pages

[1] The company Novartis has resulted from the merger of the companies Ciba-Geigy Ltd. and Sandoz Ltd. and is partial successor in business from above-named companies.

TABLE OF CONTENTS

1. APPROVAL 3
2. SUMMARY 4
3. OBJECTIVE OF THE INVESTIGATION 6
4. ORGANISATION 8
4.1. Staff and Responsibilities 8
4.2. Storing of Laboratory Data and Retention of Records 8
4.3. Dates 8
5. MATERIALS 9
5.1. Test Compounds 9
5.2. Reference Compounds 9
5.3. Animals and Husbandry 10
6. PROCEDURE 11
6.1. Determination of Initial Alkylation Potencies 11
6.2. Determination of Chemical Hydrolysis 11
6.3. Preparation of Mouse Liver Homogenates 11
6.4. Determination of Enzymatic Hydrolysis 12
6.5. Determination of Inhibition of Epoxide Hydrolases 12
6.5. Calculations 12
7. RESULTS 13
7.1. Alkylation Potency 13
7.2. Chemical Hydrolysis 13
7.3. Enzymatic Hydrolysis 14
7.4. Inhibition of Epoxide Hydrolases 14
8. CONCLUSIONS 15

1. APPROVAL

This report is being approved by the signatures of the

Sponsor:
Ciba Speciality Chemicals
Performance Polymers Division:
(Dr. H.J. Weideli)

(Date) February 3, 1997

Study Director:
(Dr. P. Sagelsdorff)

(Date) Jan 31, 1997

Head of Cell Biology:
(Dr. F. Waechter)

(Date) January 31, 1997

2. SUMMARY

Incubation of three glycidyl compounds (MY 790, CY 179 and CY 184) and two reference compounds (styrene oxide, SO, and bis(2,3-epoxycylopentyl)ether, BECPE) with the model nucleophile NBP resulted in the production of a blue-coloured alkylation product with a maximal optical absorbance at 560-580 nm. MY 790 and CY 184 showed 3 and 2 times higher alkylation activities than SO, respectively. With CY 179 and BECPE the alkylation activities were 14 and 31 times lower, respectively, than those obtained with SO.

A preincubation of the glycidyl and reference compounds in 0.1 N HCl reduced the initial alkylation activity due to hydrolysis of their epoxide moieties. The extent of this reduction reflects the susceptibility of their epoxide moieties to chemical hydrolysis. The hydrolytic stability of the investigated compounds, expressed as half-life times of their epoxide moieties in 0.1 N HCl were within a factor of 6 with the following ranking: BECPE > CY 179 ≈ MY 790 ≈ CY 184> SO. Preincubation of the test compounds in 0.01 N HCl for 1 hour did not substantially decrease the alkylation activities.

Upon preincubation with mouse liver homogenate the alkylation activities were also reduced to various extents, indicating different susceptibilities towards enzymatic inactivation. The respective half-life times of the epoxide moieties of the investigated compounds differed by a factor of 4 and decreased in the following order: CY 184 > BECPE > CY 179 > SO ≈ MY 790.

All investigated compounds inhibited the hydrolysis of *trans* stilbene oxide by mouse liver cytosolic epoxide hydrolase to about 40% of control at equimolar epoxide concentrations of the substrate *trans* stilbene oxide and the test compounds. With mouse liver microsomal epoxide hydrolase, BECPE showed the strongest effect and inhibited the hydrolysis of SO to about 35% of control at equimolar epoxide concentrations of the substrate and the test compound. Under the same conditions, CY 184 and SO inhibited microsomal epoxide hydrolase to about 50% of control and CY 179 and MY 790 to about 80% of control.

The results indicate that chemical hydrolysis of the epoxide moieties does not contribute substantially to an inactivation of the glycidyl compounds upon oral exposure in vivo, since at a concentration of 0.01 N HCl, corresponding approximately to the pH of the stomach, no significant hydrolysis of the glycidyl compounds was detectable.

In comparison with SO, which does not give any detectable DNA damage in mouse liver following i.p. administration, the alkylation activity of CY 179 was much lower and, in addition, this glycidyl ether was faster hydrolysed by mouse liver homogenate than the other investigated glycidyl ethers. Therefore, if at all, CY 179 might exhibit little genotoxic activity in vivo. In contrast, CY 184 showed a higher alkylation activity as well as a slower hydrolysis rate than SO, indicating that this compound might exhibit a certain genotoxic activity in vivo. The genotoxic activity of MY 790 is more difficult to predict, since its relative high alkylation activity is partially compensated by a relatively efficient inactivation by mouse liver homogenate.

Their minor inhibition of microsomal epoxide hydrolase in combination with their short biological half-life times suggests that combinations of CY 179 and MY 790 with other epoxides are unlikely to increase a putative genotoxicity of the co-adminstered epoxide. However, CY 184 inhibited microsomal epoxide hydrolase to a considerable extent and showed a stability in mouse liver homogenate similar to BECPE indicating that exposure to combinations of epoxides containing CY 184 at sufficiently high levels might exhibit a substantial genotoxic risk.

3. OBJECTIVE OF THE INVESTIGATION

Glycidyl esters and glycidyl ethers are used as cross-linking components in the manufacture of plastics and coatings. Chemical reactivity is a prerequisite of this commercial use, but is also implying toxicological consequences. Thus, compounds of this structure are supposed to be mutagenic in bacterial test systems. However, it is uncertain, whether or not they have a genotoxic activity *in vivo*. The epoxides could be hydrolysed by the low pH during stomach passage or, if absorbed, could be inactivated by epoxide hydrolases and or glutathione S-transferases. Therefore, epoxide compounds which are rapidly hydrolysed at low pH and/or epoxide hydrolases are supposed to have little or no genotoxic activity *in vivo*.

In addition, mixtures of epoxides with compounds that inhibit epoxide hydrolases might exhibit a considerable genotoxic risk since these compounds might prevent the inactivation of other epoxides. In this context, it has been demonstrated that, following topical administration to mice, bisphenol A diglycidyl ether only produces a measurable DNA damage in the skin upon co-administration with the well known inhibitor of microsomal epoxide hydrolase bis(2,3-epoxycylopentyl)ether, BECPE (Bentley et al. 1989, Carcinogenesis 10, pp 321-327; Bentley et al. Mutagenesis 4, pp 306).

In order to assess the genotoxic potency of structurally related glycidyl compounds and mixtures thereof, the interaction of glycidyl compounds with the model nucleophile 4-(p-nitrobenzyl-pyridine, NBP, was investigated after a preincubation with mouse liver homogenates from mice or in aqueous solution at different pH values. NBP is used as a surrogate for a DNA base and has been widely used to test the alkylation potency of different electrophiles (Preussmann et al. 1969; Arzneim. Forsch., 19, pp. 1059-1073). The alkylation reaction of NBP is analogous to the alkylation of DNA. The alkylated NBP derivative has the practical advantage, however, of forming a blue/violet chromophore, thus allowing colourimetric quantification of the product. The epoxide hydrolysis rates of the glycidyl compounds were compared to the respective values of SO, which does not give any detectable DNA damage in mouse liver following i.p administration (Cantoreggi et al. 1992, Carcinogenesis 13, pp 193-197).

Furthermore, an investigation of the inhibition of mouse liver epoxide hydrolases by glycidyl compounds was dedicated to the identification of potential 'dangerous' mixtures

in which one component might act as an inhibitor and thereby prevent the inactivation of another component by epoxide hydrolases. The inhibition of mouse liver microsomal and cytosolic epoxide hydrolases was assessed by measuring the hydrolysis rates of the standard substrates SO and *trans* stilbene oxide in the presence of different glycidyl compounds. The values were compared to the inhibition obtained with BECPE, a known inhibitor for microsomal epoxide hydrolase.

4. ORGANISATION

4.1. Staff and Responsibilities

Sponsor:

Ciba Speciality Chemicals, Performance Polymers Division.

Monitoring Scientist:

Dr. H.J. Weideli, R-1002.2.59, Tel. 7 29 84.

Study Director:

Dr. P. Sagelsdorff. R-1058.2.52, Tel. 7 61 93.

Study Investigators:

B. Heuberger, R-1058.2.50.

G. Buser, R-1058.2.48.

Research Unit:

Novartis Crop Protection Inc., Toxicology Services/Cell Biology, CH-4002 Basel, Switzerland.

4.2. Storing of Laboratory Data and Retention of Records

Novartis Crop Protection Inc., Toxicology Services/Cell Biology, CH-4002 Basel, Switzerland.

4.3. Dates

Study Initiation Date:	August, 1993
Experimental Start Date:	September, 1993
Experimental Termination Date:	March, 1994

5. MATERIALS

5.1. Test Compounds

- Bisphenol A diglycidylether (MY 790)
- Hexahydrophthalicacid diglycidylester (CY 184)
- Araldite CY 179 (CY 179)

Supplier:

Ciba Speciality Chemicals, Performance Polymers Division

5.2. Reference Compounds

- Styrene oxide (SO)
- *trans* Stilbene oxide (tSO)
- Bis(2,3-epoxycylopentyl)ether (BECPE)

Supplier:

Styrene oxide:	Fluka
trans Stilbene oxide:	Aldrich Chem. Co.
Bis(2,3-epoxycylopentyl)ether	J.M.Holland, Oak Ridge Laboratory, Oak Ridge, TN, USA)

5.3. Animals and Husbandry

Supplier:

Novartis Crop Protection Inc., breeding station, Stein, Switzerland.

Species:

Male Tif:MAGf mice

Acclimatisation period:

One week

Cages:

Macrolon cages

Air conditions:

Temperature: 22±3°C; Humidity: 40-70%

Illumination Time:

6 a.m. to 6 p.m.

Food:

Nafag 890 (Nafag, Gossau, Switzerland), ad libitum

Water:

Tap water ad libitum

6. PROCEDURE

6.1. Determination of Initial Alkylation Potencies

The test compounds as well as the reference compounds were dissolved in acetone/ethylene glycol (1+2 by volume) to yield concentrations of 0.4 mM and 2 mM. 2.5 ml of these solutions were mixed with 2.0 ml phosphate buffer (100 mM, pH 7.2) and 0.5 ml 0.12 M p-nitrobenzyl pyridine (NBP) in acetone. After different time periods, 0.8 ml were withdrawn and made alkaline by the addition of 0.2 ml triethylamine. The optical density at 580 nm was recorded immediately.

6.2. Determination of Chemical Hydrolysis

The test compounds as well as the reference compounds, dissolved in acetone/ethylene glycol (1+2 by volume), were mixed with an equal amount of 0.2 N HCl or 0.02 N HCl, to give a final concentration of 2 mM. After different preincubation times at 37 °C aliquots of 530 µl of the hydrolysate were diluted with 530 µl acetone/ethylene glycol (1+2 by volume) and neutralised by the addition of 110 µl 0.5 M phosphate buffer, pH 7.2, and 265 µl 0.2 N NaOH or 0.02 N NaOH. Control incubations were made in 100 mM phosphate buffer, pH 7.2. The alkylation activity of the neutralised hydrolysate was determined by incubation with 160 µl NBP-solution (0.12 M in acetone) for 1 hour. Due to the low alkylation activity of CY 179 and BECPE incubations of these glycidyl compounds with NBP were carried out for 24 hours.

6.3. Preparation of Mouse Liver Homogenates

Mouse livers were homogenised in 3 to 5 volumes 250 mM sucrose, 10 mM Tris/HCl, pH 7.2, in a Elvehjem type teflon potter. The homogenates were centrifuged for 15 min at 1000 g and the supernatants were used for the incubations. The protein content of the homogenates was determined using the BCA protein assay (Pierce, IL, USA).

6.4. Determination of Enzymatic Hydrolysis

The test compounds as well as the reference compounds were dissolved in DMSO to give a final concentration of about 2 mM. 30 µl of these solutions were incubated with mouse liver homogenate (about 7 mg protein/ml) in a final volume of 0.6 ml. After different time periods the proteins were precipitated by the addition of 2 volumes acetone and by centrifugation. 200 µl of the supernatant was assessed for alkylating activity by incubation with 1 ml NBP (57.6 mM) in acetone/ethylene glycol/100 mM phosphate buffer, pH 7.2 (18 +41+41 by volume) for 24 hours.

6.5. Determination of Inhibition of Epoxide Hydrolases

The epoxide hydrolysis of *trans* stilbene oxide and SO, standard substrates for cytosolic and microsomal epoxide hydrolases, respectively, was assessed in the presence and in the absence of the test compounds as well as the reference compounds as described by Waechter et al. 1988 [Biochem. Pharmacol. 37, 3897-3903].

6.5. Calculations

Following incubation at low pH values or in the presence of tissue homogenates, the alkylation activities of the test compounds as well as the reference compounds were decreased. The remaining activities, expressed in alkylation equivalents, were found to decrease exponentially with increasing times:

$$A(t) = A_0 \cdot e^{-(t/T)\ln 2} \qquad (1)$$

Where: A(t): Remaining alkylation equivalents after preincubation for time t
A_0: Initial alkylation equivalents
T: Half-life time (preincubation time necessary to inactivate 50% of the initial alkylation equivalents)

For the calculation of the half-life time, T, equation (1) was linearised to:

$$-\ln(A/A_0) = (t/T)\cdot \ln 2 \qquad (2)$$

7. RESULTS

7.1. Alkylation Potency

Incubation of the glycidyl and the reference compounds with the model nucleophile NBP resulted in the production of a blue-coloured alkylation product with a maximal optical absorbance at 560-580 nm. The increase of optical density was linear with time and the respective test-compound concentration (Table 1, Figure 1). A linear regression of the optical density at 580 nm vs. incubation time was used to calculate the alkylation activity as increase of the optical density at 580 nm per minute. The alkylation activities of the investigated epoxides span over two orders of magnitude. MY 790 and CY 184 showed 3 and 2 times higher alkylation activities than SO, respectively. With CY 179 and BECPE, the alkylation activities were 14 and 31 times lower, respectively, than those obtained with SO (Table 1, Figure 2).

7.2. Chemical Hydrolysis

A preincubation of the glycidyl and reference compounds in 0.1 N HCl reduced the initial alkylation activity due to hydrolysis of their epoxide moieties (Figure 3). This reduction of the alkylation activity reflects the susceptibility of the epoxide moieties of the investigated compounds to chemical hydrolysis. Preincubation of the test compounds as well as of the reference compounds in 0.01 N HCl up to 1 hour did not substantially decrease the alkylation activities (data not shown). Under the assumption of a first order kinetics for chemical hydrolysis, a linear regression of the logarithmically transformed data allowed the calculation of the half-life times of the epoxide moieties in 0.1 N HCl (Table 2, Figure 4). The half-life times of the epoxides moieties of the investigated compounds in 0.1 N HCl differed by a factor of 6 with the following ranking: BECPE > CY 179 ≈ CY 184 ≈ MY 790 > SO.

7.3. Enzymatic Hydrolysis

Upon preincubation with mouse liver homogenates the alkylation activities were also reduced. The time course of the inactivation of the investigated compounds is presented in Figure 5. A logarithmic presentation of the data suggested first order kinetics. This allowed the calculation of the half-life times of the epoxide moieties of the test compounds in the presence of mouse liver homogenate (Table 3, Figure 6). The half-life differed by a factor of 4 and decreased in the following order: CY 184 > BECPE > CY 179 > SO ≈ MY 790.

7.4. Inhibition of Epoxide Hydrolases

All investigated compounds inhibited the hydrolysis of *trans* stilbene oxide by cytosolic epoxide hydrolase to about 40% of its control value at equimolar concentrations of the substrate and the test compounds (Table 4, Figure 7). With mouse liver microsomal epoxide hydrolase BECPE showed the strongest effect and inhibited the hydrolysis of SO to about 35% of control at equimolar epoxide concentrations of the substrate and the test compounds. Under the same conditions CY 184 and SO inhibited microsomal epoxide hydrolase to about 50% of control and CY 179 and MY 790 to about 80% of control (Table 4, Figure 8).

8. CONCLUSIONS

The results clearly show that SO, which does not give any detectable DNA damage in mouse liver after i.p. administration, exhibits a moderate alkylation potency, but is hydrolysed relatively fast by 0.1 N HCl and in mouse liver microsomes.

Preincubation of the test compounds in 0.01 N HCl for 1 hour did not substantially decrease the alkylation activities. Since the pH of the stomach corresponds to a concentration of about 0.01 N HCl, chemical hydrolysis of the epoxide moieties does not contribute substantially to an inactivation of the glycidyl compounds after oral administration in vivo.

In comparison with SO, the alkylation activity of CY 179 was much lower and, in addition, this glycidyl ether was faster hydrolysed by mouse liver homogenate than the other investigated glycidyl ethers. Therefore, if at all, CY 179 might exhibit little genotoxic activity in vivo. In contrast, CY 184 showed a higher alkylation activity as well as a slower hydrolysis rate than SO indicating that CY 184 might exhibit a certain genotoxic activity in vivo. The genotoxic activity of MY 790 is more difficult to predict since its relative high alkylation activity is partially compensated by a relatively efficient inactivation by mouse liver homogenate.

The minor inhibition of microsomal epoxide hydrolase in combination with their short biological half-life times suggests that combinations of CY 179 and MY 790 with other epoxides are unlikely to increase a putative genotoxicity of the co-administered epoxide. However, CY 184 inhibited microsomal epoxide hydrolase to a considerable extent and showed a stability in mouse liver homogenate similar to BECPE indicating that exposure to combinations of epoxides with CY 184 at sufficiently high levels might exhibit a substantial genotoxic risk.

Table 1

Alkylation of NBP by different epoxides. The investigated compounds (epoxide concentration: 1 and 0.2 mM) were incubated with NBP (12 mM) for various time periods. A linear regression of the optical density at 580 nm vs. incubation time was used to calculate the increase of the optical density per minute.

	oD 580 nm/min	
Epoxide Compound	1 mM	0.2 mM
MY 790	8.50E-03	1.44E-03
CY 184	6.12E-03	1.04E-03
CY 179	1.99E-04	6.25E-05
BECPE	8.93E-05	n.d.
SO	2.80E-03	4.97E-04

n.d. not detectable

Table 2

Half lives (mean of 3 experiments ± S.D.) of different epoxides upon hydrolysis in 0.1 N HCl. The investigated compounds (epoxide concentration 2 mM) were incubated in 0.1 N HCl for different time periods. After neutralisation with 1 volume 0.1 N NaOH, NBP (final concentration: 12 mM) was added and the optical density at 580 nm was recorded after 1 hour (24 hours for CY 179 and BECPE). Assuming first order kinetics for the hydrolysis of the epoxide moiety, a linear regression of the logarithm of the optical density vs. hydrolysis time was used to calculate the half lives of the epoxy moieties in 0.1 N HCl.

Epoxide Compound	Chemical Half Life [min]
MY 790	10.3 ± 0.9
CY 184	12.9 ± 0.7
CY 179	11.7 ± 6.0
BECPE	27.2 ± 16.3
SO	4.4 ± 4.1

Table 3

Half life (mean of 3 experiments ± 1 S.D.) of different epoxides upon hydrolysis in mouse liver homogenate. The investigated compounds (epoxide concentration 2 mM) were incubated with mouse liver homogenates (7 mg protein/ml) for different time periods. After precipitation of the proteins with 2 volumes methanol, NBP (final concentration: 12 mM) was added to the supernatant and the optical density at 580 nm was recorded after 24 hours. A linear regression of the logarithm of the optical density vs. hydrolysis time was used to calculate the half lives of the epoxy moieties in the presence of mouse liver homogenates.

Epoxide Compound	Enzymatic Half Life [min]
MY 790	11.2 ± 1.8
CY 184	54.8 ± 13.2
CY 179	33.4 ± 10.9
BECPE	46.1 ± 0.8
SO	13.6 ± 5.4

Table 4

Inhibition of mouse microsomal and cytosolic epoxide hydrolase by different epoxides. Hydrolysis of *trans*-[^{14}C]stilbene oxide (cEH) and [^{14}C]SO (mEH) by mouse liver cytosolic and microsomal fractions, respectively, was determined in the presence and absence of equimolar amounts of the epoxides.

	Activity [nmol/min/mg Protein]	
Epoxide Compound	microsomal EH	cytosolic EH
MY 790	2.54 ± 0.22	1.23 ± 0.06
CY 184	1.70 ± 0.15	1.14 ± 0.26
CY 179	2.45 ± 0.30	1.20 ± 0.02
BECPE	0.93 ± 0.10	1.04 ± 0.31
SO	1.83 ± 0.13	1.21 ± 0.13
Cont	3.23 ± 0.31	3.03 ± 0.71

Figure 1

Alkylation of NBP by different epoxides. The investigated compounds (epoxide concentration: 1 mM) were incubated with NBP (12 mM) for various time periods.

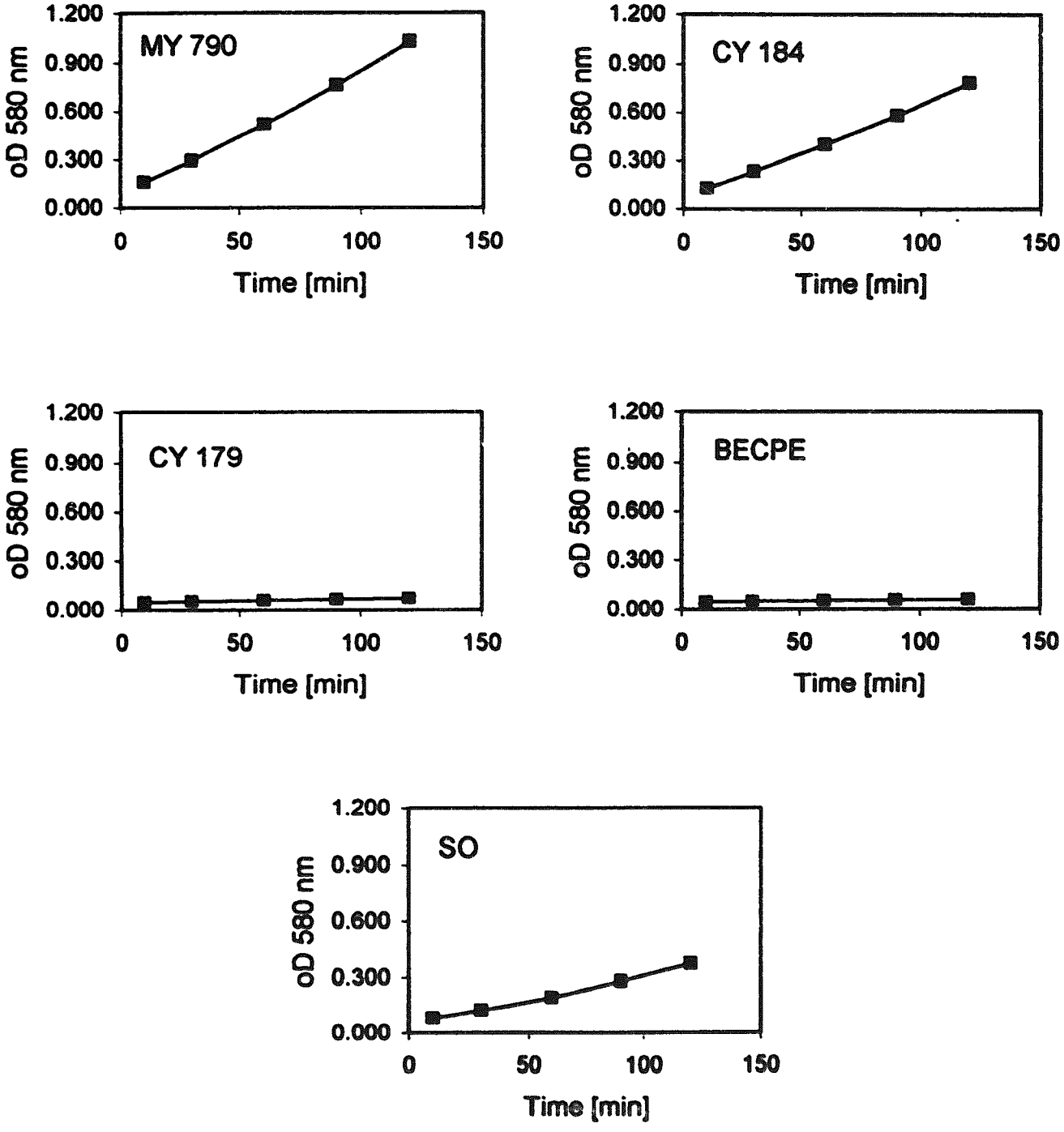

Figure 2

Alkylation activity of different epoxides. The investigated compounds (epoxide concentration: 1 mM) were incubated with NBP (12 mM) for various time periods. A linear regression of the optical density at 580 nm vs. incubation time was used to calculate the increase of the optical density per minute.

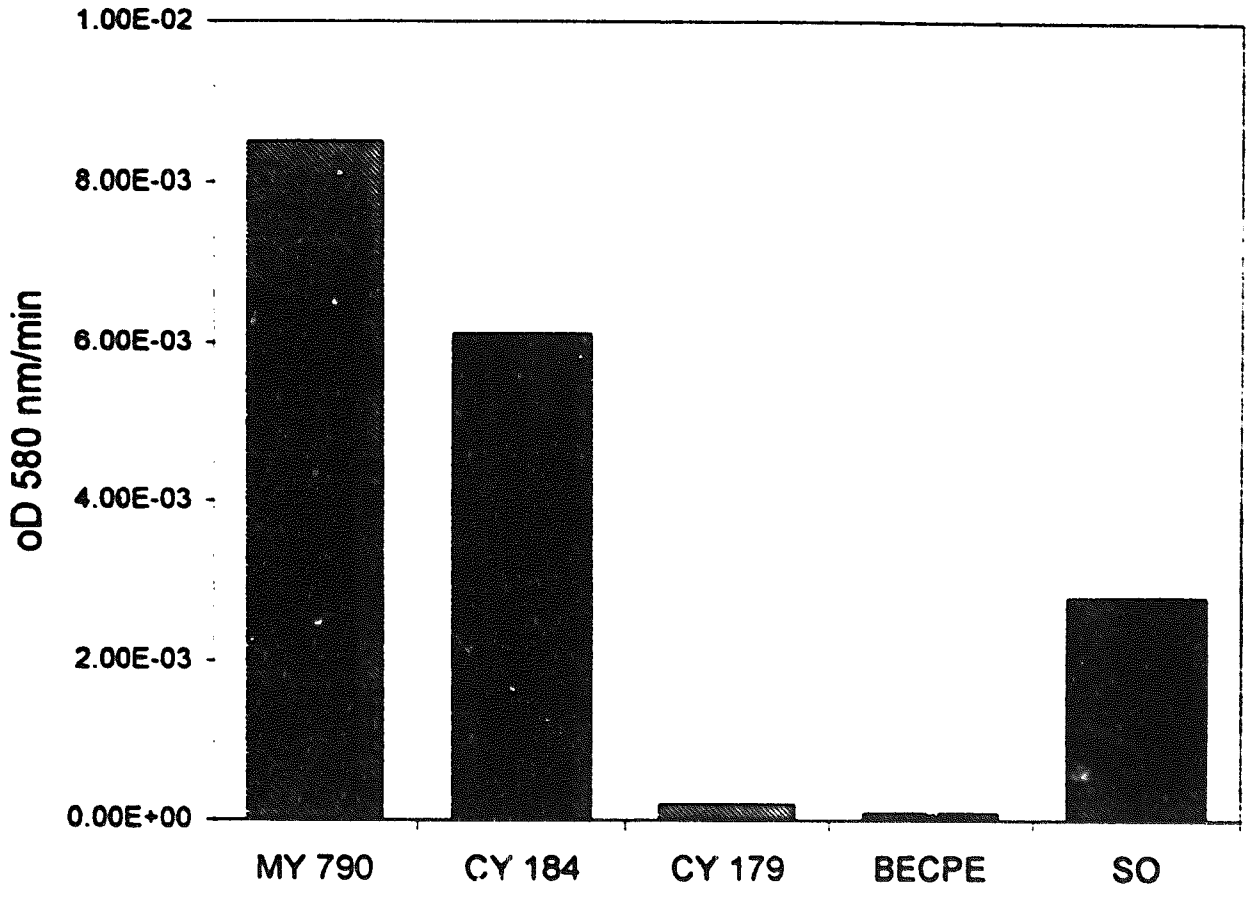

Figure 3

Alkylation of NBP by different epoxides after preincubation in 0.1 N HCl for different time periods. The investigated compounds (epoxide concentration 2 mM) were incubated in 0.1 N HCl for different time periods. After neutralisation with 1 volume 0.1 N NaOH, NBP (final concentration: 12 mM) was added and the optical density at 580 nm was recorded after 1 hour (24 hours with CY 179 and BECPE).

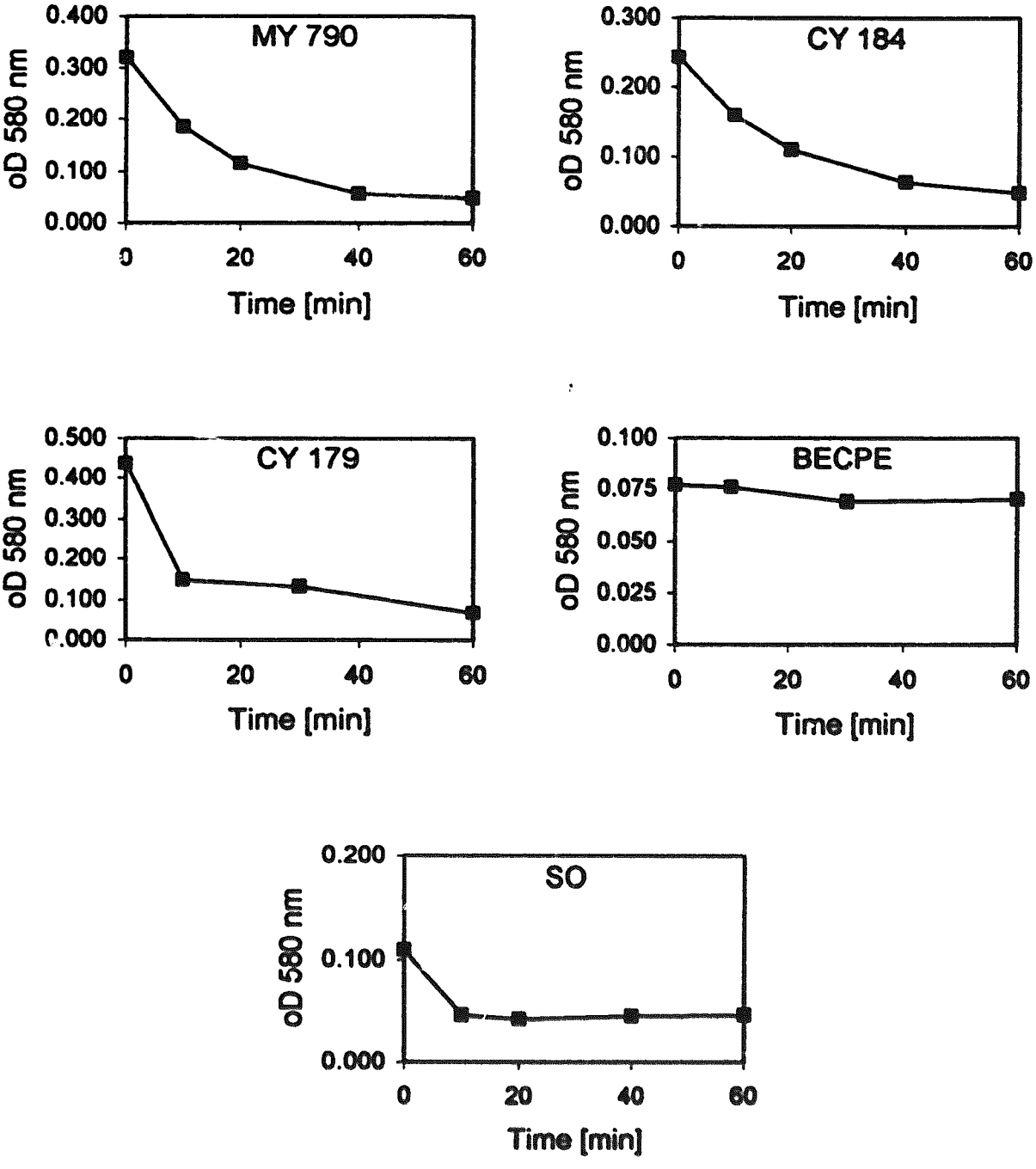

Figure 4

Half lives of different epoxides upon hydrolysis in 0.1 N HCl. The investigated compounds (epoxide concentration 2 mM) were incubated in 0.1 N HCl for different time periods. After neutralisation with 1 volume 0.1 N NaOH, NBP (final concentration: 12 mM) was added and the optical density at 580 nm was recorded after 1 hour (24 hours with CY 179 and BECPE). Assuming first order kinetics for the hydrolysis of the epoxide moiety, a linear regression of the logarithm of the optical density vs. hydrolysis time was used to calculate the half lives of the epoxy moieties in 0.1 N HCl.

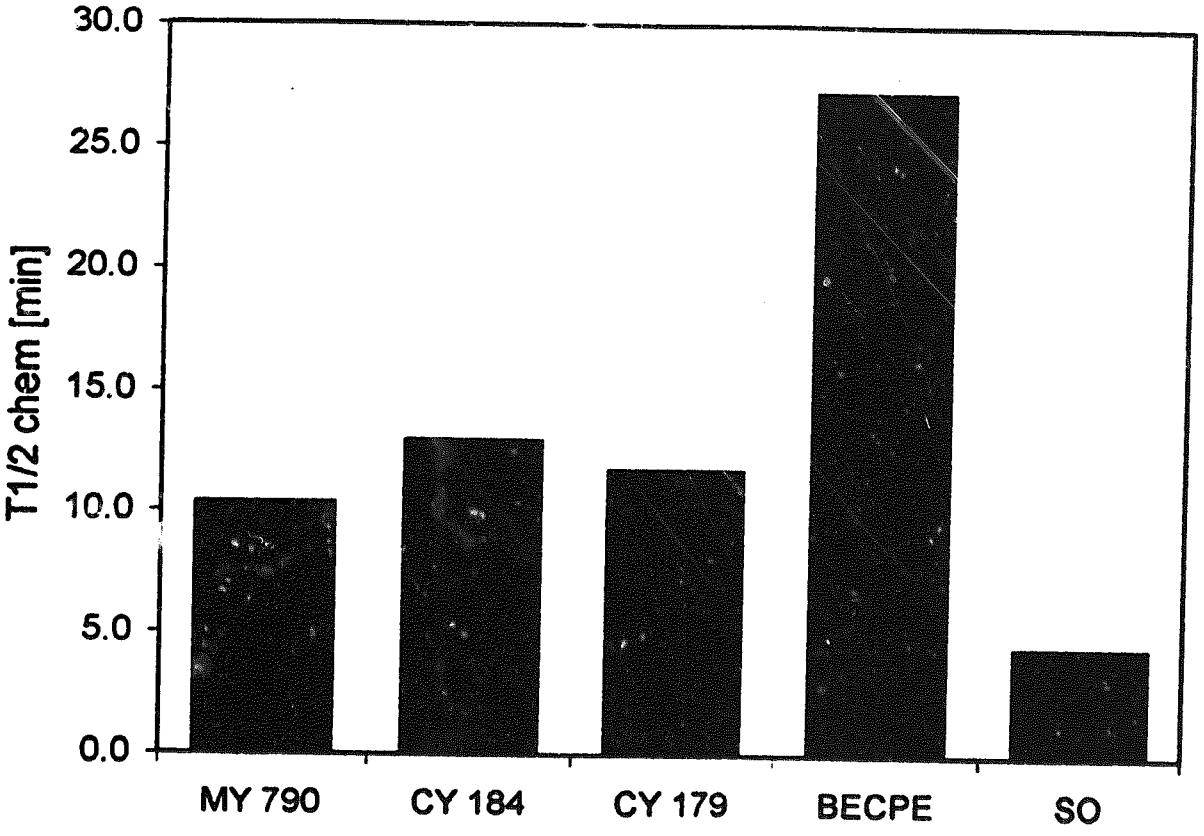

Figure 5

Alkylation of NBP by different epoxides after preincubation in mouse liver homogenates for different time periods. The investigated compounds (epoxide concentration 2 mM) were incubated with mouse liver homogenates (5-10 mg protein/ml) for different time periods. After precipitation of the proteins with 2 volumes methanol, NBP (final concentration: 12 mM) was added to the supernatant and the optical density at 580 nm was recorded after 24 hours.

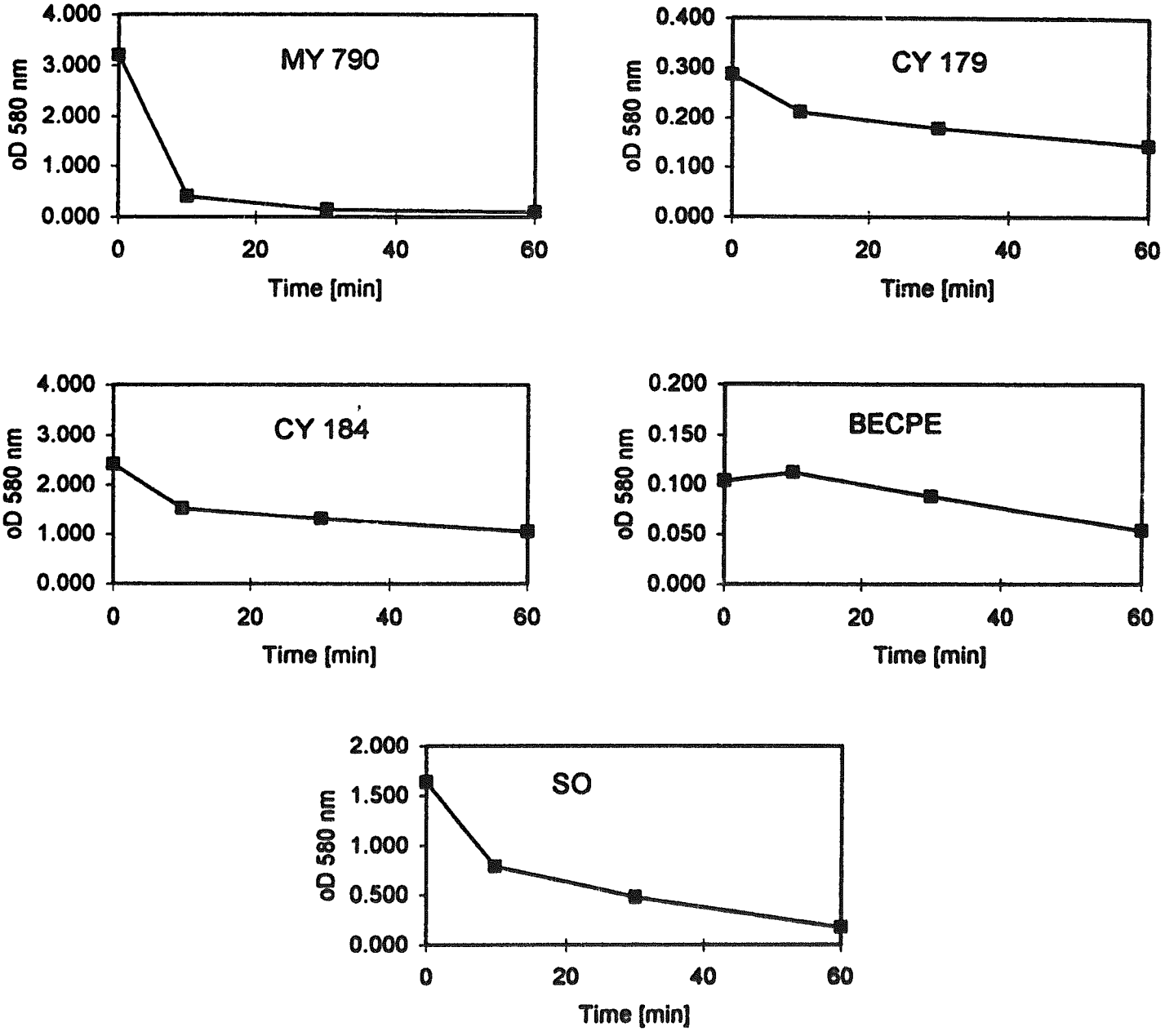

Figure 6

Half life of different epoxides upon hydrolysis in mouse liver homogenate. The investigated compounds (epoxide concentration 2 mM) were incubated with mouse liver homogenates (7 mg protein/ml) for different time periods. After precipitation of the proteins with 2 volumes methanol, NBP (final concentration: 12 mM) was added to the supernatant and the optical density at 580 nm was recorded after 24 hours. A linear regression of the logarithm of the optical density vs. hydrolysis time was used to calculate the half lives of the epoxy moieties in the presence of mouse liver homogenates.

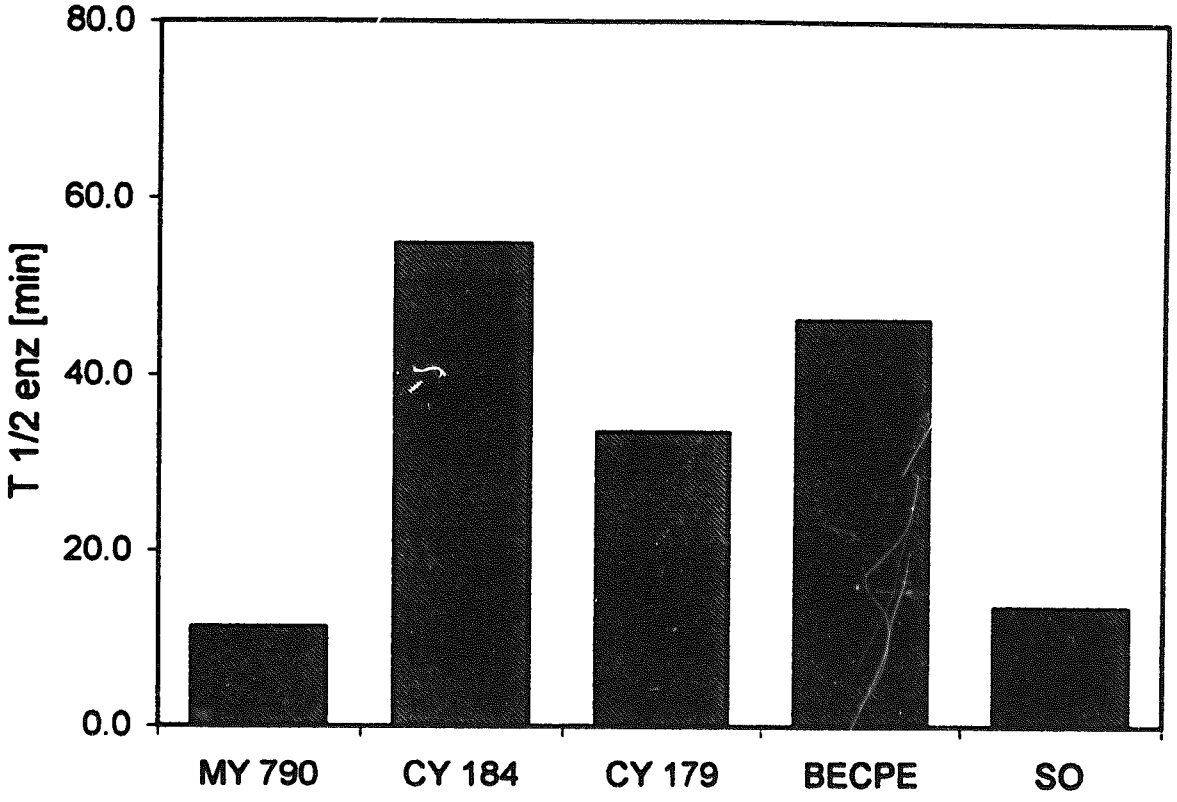

Figure 7

Inhibition of mouse cytosolic epoxide hydrolase by different epoxides. Hydrolysis of *trans*-[^{14}C]stilbene oxide by mouse liver cytosolic fractions (1-3 mg protein/ml) was determined in the presence and absence of epoxide-equimolar amounts of the investigated compounds. The activity of cytosolic epoxide hydrolase in the presence of the investigated compounds was expressed as percentage of the respective activity in the absence of any inhibitor.

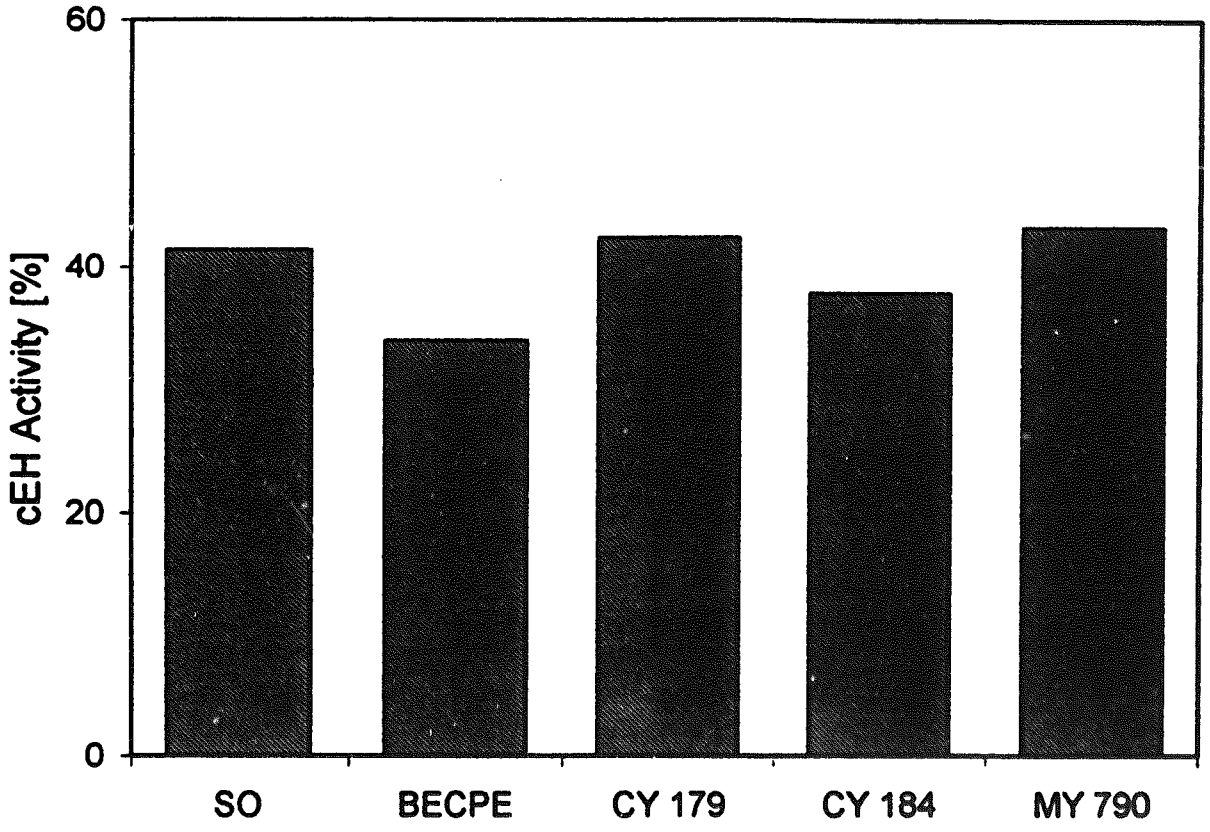

Figure 8

Inhibition of mouse microsomal epoxide hydrolase by different epoxides. Hydrolysis of [^{14}C]SO by mouse liver microsomal fractions (3-6 mg protein/ml) was determined in the presence and absence of epoxide-equimolar amounts of the investigated compounds. The activity of microsomal epoxide hydrolase in the presence of the investigated compounds was expressed as percentage of the respective activity in the absence of any inhibitor.